REMARKS

Reconsideration and allowance are respectfully requested.

After the entrance of the present amendments, claims 1 and 5-22 are pending. Elected claims 1 and 3-14 have been examined on the merits; non-elected claim 2 was withdrawn from consideration by the Examiner. Applicants have canceled the non-elected claim without prejudice and reserve the right for future prosecution of that subject matter.

The amendments are supported by the original disclosure and, thus, no new matter has been added. If the Examiner should disagree, however, she is respectfully requested to point out the challenged limitation with particularity in the next Action so support may be cited in response. The amendments should not be construed as an indication of Applicants' agreement with or acquiescence in the rejections of record.

Claims 3 and 5-7 were objected to. The claims recite sequence identifiers for the elected invention. The adjectives "transformed" and "transgenic" have been used in claims 5-6 and 7, respectively. In claim 7, "an" has been inserted. Withdrawal of the claim objectives is respectfully requested.

35 U.S.C. 112 – Written Description

The specification must convey with reasonable clarity to persons skilled in the art that applicant was in possession of the claimed invention as of the filing date sought. See *Vas-Cath v. Mahurkar*, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991). However, the Patent Office has the initial burden of presenting evidence or a reason why persons of ordinary skill in the art would not have recognized such a description of the claimed invention in the original disclosure. See *In re Gosteli*, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989).

Claims 1 and 4-11 were rejected under Section 112, first paragraph, because they allegedly contain "subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention." Applicants respectfully disagree.

According to the Examiner, the "claimed [DNA] genus encompasses a multitude of different nucleotide sequences and [corresponding] proteins, including those yet to be discovered" (page 3 of the Office Action mailed on August 13, 2002). In order to expedite the prosecution, Applicants have amended the claims. In brief, the amendments are as follows:

(i) The limitations of claim 3 have been incorporated into claim 1 to more clearly define the claimed invention.

(ii) Variation has been limited to up to ten conservative amino acid substitutions in part (c).

(iii) The sequence identity of the protein has been limited to "at least 80%" in part (d).

Therefore, claim 1 now includes only a limited number of variants. The genus is not widely variant; it permits only slight variation and allows only a small degree of structural differences among its members. The description on pages 11-14 of the specification shows that a representative number of nucleotide sequences within the claimed genus were in Applicants' possession.

Claim 7 was rejected under Section 112, first paragraph, because it allegedly contains "subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention." Applicants respectfully disagree.

According to the Examiner, "there are insufficient relevant identifying characteristics to allow one skilled in the art to predictably determine the genotypic or phenotypic characteristics of the offspring or clone plant obtained" (page 4 of the Office Action mailed on August 13, 2002). Amended claim 1 (from which claim 7 ultimately depends) now recites the genotype conferred by the DNA. Therefore, since the genotypic features of the transformed plant are now identified, the genotypic features of its progeny or clone would also be apparent to one of ordinary skill in the art. Furthermore, claim 7 has been amended to expressly require the presence of the DNA of claim 1.

Withdrawal of the written description rejections made under Section 112, first paragraph, is respectfully requested for the above reasons.

35 U.S.C. 112 – Enablement

The Patent Office has the initial burden to question the enablement provided for the claimed invention. M.P.E.P. § 2164.04, and the cases cited therein. It is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. *In re Marzocchi*, 169 USPQ 367, 370 (C.C.P.A. 1971). Specific technical reasons are always required. See M.P.E.P. § 2164.04.

Claims 1 and 3-14 were rejected under Section 112, first paragraph, because it was alleged that the specification does not reasonably provide enablement for the full scope of the invention. Applicants respectfully disagree.

According to the Examiner, the specification enables (i) *Arabidopsis* AtNCED3 DNA comprising SEQ ID NO:5 encoding polypeptide of amino acid sequence set forth in SEQ ID NO:6 and (ii) a method of increasing/decreasing drought stress tolerance only in *Arabidopsis* plants by transforming them with the *Arabidopsis* AtNCED3 DNA comprising SEQ ID NO:5 encoding polypeptide of amino acid sequence set forth in SEQ ID NO:6, but it does not enable other DNA molecules or for increasing/decreasing drought stress tolerance in any other plant species. The Examiner alleges that undue experimentation would be required to determine:

(i) how to express a DNA encoding a protein having neoxanthin cleavage activity such that tolerance to stresses other than drought is increased/decreased,

(ii) which amino acid replacements, deletions, additions or insertions need to be made in the polypeptide sequences comprising SEQ ID NO:6, such that the resulting altered polypeptide retains neoxanthin cleavage activity such that tolerance to stresses drought is increased/decreased,

(iii) which DNA molecules within the scope of the instant claims would encode a polypeptide which retains the characteristics of a protein that has neoxanthin cleavage activity,

(iv) which species of plant other than *Arabidopsis* could be employed to express the claimed DNA,

(v) how much to increase or decrease the expression of the DNA that ultimately would result in an increase/decrease in drought resistance, and

(vi) with respect to claims 9 and 10, how the same DNA could both increase and decrease the amount of abscisic acid or stress tolerance.

Regarding item (i), Applicants teach on page 38, lines 11-15, of the specification that their invention would be equally useful for stresses other than drought:

> "As a result, it was found the AtNCED3 gene expression was strongly induced by drought, high salt concentration, and cold condition. Heat condition did not induce the expression. In addition, for ABA treatment or water treatment, the induction of the AtNCED3 gene expression was not detected (Fig. 12)."

Since Applicants' specification clearly teaches that the AtNCED3 gene is induced by stresses other than drought (e.g., high salt concentration and cold conditions), the skilled person would readily recognize that the instant invention is equally effective for improving tolerance to such other stresses as well. Such an effectiveness can be easily shown by routine experimentation as taught in this specification using some other stress (such as high salt concentrations and cold conditions) in place of drought stress. Such experimentation could hardly be viewed as undue.

Regarding item (ii), the claimed genus of DNA is focused on a limited number of sequences. Specifically, claim 1 is now limited to a DNA encoding a protein of SEQ ID NO:6, a protein encoded by a DNA that hybridizes under stringent conditions with a DNA of SEQ ID NO:5; a protein having the amino acid sequence of SEQ ID NO:6 with up to ten conservative amino acid substitutions; or a protein having an amino acid sequence that is at least 80% identical to the sequence set forth in SEQ ID NO:6. New claims 15-18 further limit the claimed DNA molecule to that encoding a protein having an amino acid sequence that is at least 90%, 95%, 99% or 100% identical to SEQ ID

NO:6, respectively. Therefore, the number of proteins that can fulfill the structural and functional limitations as set forth in the claims as amended are quite limited, and a skilled person could isolate a DNA encoding such a protein without undue experimentation.

Regarding item (iii), the claims have been amended such that they do not include "all modifications" but only include a narrow subset thereof. Naturally, the subset of modified proteins claimed would be expected to retain the function of the wild-type protein of SEQ ID NO:6 and this is required by the terms of claim 1. Furthermore, operable embodiments may be easily distinguished from non-operable embodiments (i.e., which modified proteins have neoxanthin cleavage activity and which do not) using the enzyme assay described in Example 5 of the specification.

Regarding item (iv), the Examiner admits that "one of ordinary skill in the art could readily make transgenic plants expressing other DNAs." Her concern appears to stem from the breadth of DNA encompassed by the claimed invention. Accordingly, the claim amendments and above arguments address this objection.

Regarding item (v), the identification of optimal expressions levels needed to achieve a desired result is certainly within the skill of the art and involves merely routine experimentation. It is the quality and not the quantity of experimentation that must be analyzed when determining undue experimentation. No evidence to the contrary has been made of record.

Regarding item (vi), the Examiner questions how the same DNA could both increase and decrease the amount of abscisic acid or stress tolerance. Claims should be read in light of the specification and, in this case, the specification adequately describes both a decrease and increase in drought stress (pages 3-4):

> "The present inventors first produced a transgenic plant of Arabidopsis using AtNCED3, a neoxanthin cleavage enzyme gene. The AtNCED3 gene was ligated downstream of 35S promoter in a vector for introducing a gene into plant cells (pBE2113N) in the directions of sense (an overexpression type) or antisense (an expression inhibition type) and introduced the vector into Arabidopsis by the vacuum infiltration method. Evaluation of drought tolerance of the prepared transgenic plants revealed that stress tolerance in the overexpressed plants was significantly increased compared with that in their parent lines. In contrast, in the

> expression-inhibited lines into which the antisense was introduced, stress tolerance was reduced (Figs. 15 and 16). In such a manner, the present inventors found that actually the transgenic plant into which the neoxanthin cleavage enzyme gene is introduced significantly increased stress tolerance and stress tolerance can be significantly reduced by decreasing the expression of the gene to complete the present invention".

In other words, it is clear that stress tolerance would increase if the AtNCED3 gene is overexpressed, and stress tolerance would decrease when the gene expression is inhibited. Furthermore, the Examples adequately show how this is done.

In sum, the scope of the claims as amended is commensurate with the scope of enablement of the present specification. Withdrawal of the enablement rejection made under Section 112, first paragraph, is respectfully requested because it would not require undue experimentation for a person of skill in the art to make and use the claimed invention.

35 U.S.C. 112 – Definiteness

Claims 1 and 3-14 were rejected under Section 112, second paragraph, as being allegedly "indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention." Applicants respectfully disagree.

In accordance with the Examiner's suggestion, "a" has been deleted from claims 1 and 8. Claims 3-4 have been canceled and renders this rejection moot as applied to those claims.

Claim 3 allegedly fails to define what conditions would yield the claimed DNA, as one skilled in the art utilizes hybridization conditions having different stringency. The test for indefiniteness is whether one of ordinary skill in the art would understand the metes and bounds of the claim, when read in light of the specification and in the context of the prior art. Thus, claim language cannot be analyzed in a vacuum, but must be interpreted in light of the specification, the teachings of the prior art, and the reasonable interpretation given by one of ordinary skill in the art. In this case, the specification provides clear guidance as to what may be considered "stringent conditions." For example, page 14 of the specification teaches:

> "Hybridization can be performed under stringent condition by following, for example, the method described in reference (Sambrook, J., et al., "Molecular Cloning: A Laboratory Manual" 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) using a [^{32}P]-labeled DNA prepared by using a random prime method as a probe. A DNA is blotted to a nylon membrane and hybridized with a [^{32}P]-labeled fragment, for example, in a hybridization solution containing 30%, preferably 50% formamide, 6X SSC, 5X Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA, at 37°C, preferably at 42°C. Under a stringent condition, washing is, for example, in 1X SSC, 1% SDS (room temperature), for 15 min twice, preferably (more stringent) in 0.5X SSC, 0.5% SDS (37°C), for 15 min twice, and more preferably (further stringent) 0.1X SSC, 0.1% SDS (60°C) for 15 min twice, and subjected to autoradiography. By "hybridizes under stringent conditions" is meant specific and non-covalent equilibrium binding by base-pairing to an immobilized reference nucleic acid under the above conditions."

To expedite prosecution, however, the specific hybridization conditions have been recited in amended claim 1, which incorporates the limitations of original claim 3.

Cancellation of claim 4 renders moot the rejection that it is indefinite for reciting the term "derived" therein..

In accordance with the Examiner's suggestion, "comprising" has been substituted in claims 5 and 13.

In accordance with the Examiner's suggestion, the term "isolated DNA" has been substituted in claim 8 and in other claims which recite the DNA of claim 1.

The term "its wild type" in claims 8-10 has been clarified to show that the comparison is to the wild type of the transgenic plant.

The Examiner alleges that the step of increasing or decreasing stress tolerance in a plant has been omitted. Although this step is implicit in claim 14 and conventional in the art, the claim has been amended to explicitly recite it.

In view of the above amendments and arguments, Applicants submit that the amended claims meet the threshold requirement of clarity and precision and are therefore in compliance for definiteness under 35 U.S.C. 112, second paragraph. Hence, Applicants respectfully request that the rejection to these claims under 112, second paragraph be reconsidered and withdrawn.

35 U.S.C. 101 –Utility

Claims 1 and 3-11 were rejected under 35 U.S.C. 101 because the claimed invention is allegedly directed towards non-statutory subject matter since "[a] DNA encoding a protein having neoxanthin cleavage activity for improving stress tolerance in a plant would occur in nature" (page 10 of the Office Action mailed on August 13, 2002). In other words, the claims read on "a product of nature." In accordance with the Examiner's suggestion, claim 1 has been amended to recite an "isolated DNA" and thereby overcomes this rejection.

35 U.S.C. 102 – Novelty

Claims 1 and 3 were rejected under Section 102(b) as allegedly being anticipated by Tan et al. (Accession No. 2MU95953, 1997). Applicants respectfully disagree.

Claim 3 has been canceled and its limitations incorporated into claim 1. Furthermore, the phrase "replaced, deleted, added, and/or inserted" has been deleted from the claims. Additionally, the claims are now directed to DNA that encodes a protein having neoxanthin cleavage activity and an amino acid sequence that is: (a) SEQ ID NO:6; (b) encoded by a DNA that hybridizes under stringent conditions with SEQ ID NO:5; (c) SEQ ID NO:6, with 10 or fewer conservative amino acid substitutions, or (d) a sequence at least 80% identical to SEQ ID NO:6. Therefore, the VP14 protein of Tan et al., which has only a 64% sequence identity to SEQ ID NO:6, is clearly excluded from the scope of claim 1. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. 102(b) to claim 1 are respectfully requested.

Claim 7 was rejected under Section 102(b) as allegedly being anticipated by or, in the alternative, under Section 103(a) as allegedly obvious under Jaglo-Ottoson et al (Science 280:104-106, 1998). Applicants respectfully disagree.

Amended claim 1 (from which claim 7 ultimately depends) explicitly recites the genotype. Since the genotypic features of the transformed plant are now identified, the genotypic features of its progeny or clone would also be apparent to one of ordinary skill in the art. This feature is absent from Jaglo-Otttosen et al., who disclose a transgenic *Arabidopsis* plant comprising a DNA encoding the transcriptional activator CBF1 (page

104, col. 3, 1st paragraph). Hence, Jaglo-Otttosen et al. fail to disclose each and every element of claim 7, and therefore, cannot anticipate it.

Furthermore, the Jaglo-Otttosen et al. disclosure and the claimed invention are fundamentally different. Stress tolerance is acquired in Jaglo-Otttosen et al. by introducing a transcription factor (CBF1), whereas the instant invention introduces a gene for an enzyme (NCED) that functions in the synthesis of an abscisic acid (ABA), a plant hormone, and improves stress tolerance by increasing the ABA amount through up-regulating NCED expression. Thus, the instant invention is clearly not suggested by Jaglo-Otttosen et al. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. 102(b) to claim 7 are respectfully requested.

35 U.S.C. 103 – Nonobviousness

Claims 1, 3, 5-11 and 13 were rejected under Section 103(a) as allegedly being unpatentable over Tan et al. (Accession No. 2MU95953, 1997). Applicants respectfully disagree.

Although Tan et al. disclose a DNA which encodes a protein having neoxanthin cleavage activity, prior to the instant invention it had "*not been confirmed whether introduction of the DNA encoding this enzyme into a plant actually leads to ABA accumulation and improvement of tolerance against stresses without a grave effect to plant's growth*" (page 9, lines 12-16, of the specification). As Applicants further explain:

> "Exogenous treatment with ABA causes, for example, growth inhibition in many plants. In a seed, it is known that ABA also causes growth inhibition (germination inhibition) (Takahashi, N. and Masuda, Y. (eds), "Plant Hormone Handbook (The Last)," Baifukan, Japan, pp. 78-160; and references cited therein). Increase in ABA level brings about various damages to plants. There has been no report whether excessive production of ABA by an exogenous gene leads to acquirement of stress tolerance or not. The conventional experimental procedures for exogenous treatment with ABA require the treatment at high concentration, which strongly inhibits the growth and has prevented accurate evaluation of tolerance. Furthermore, experiments of exogenous treatments have not identified that an appropriate level of ABA ensures normal growth and acquirement of tolerance. By obtaining ABA biosynthesis gene and creating a transgenic plant using this gene, the

present inventors have first confirmed that stress tolerance in a plant can be improved."

Since the damaging effects of exogenous ABA treatment were well known, one of ordinary skill in the art would not be motivated to isolate and express a gene involved in ABA synthesis, merely by the teachings of Tan et al. Thus, whether introduction of such a gene would enhance stress tolerance without damaging plant growth could not have been reasonably expected by one of ordinary skill in the art without actual experimental verification, which was done, for the first time, by the present inventors. This fact alone proves that the instant invention could not have been obvious from Tan et al.

Furthermore, as discussed above, the claims as amended require a DNA distinct from that disclosed by Tan et al. There is nothing in the Tan et al. disclosure to suggest modification to the sequence of the disclosed neoxanthin cleavage protein or the DNA encoding same to fall within the scope of the amended claim.

Claims 1 and 5-14 were also rejected under Section 103(a) as allegedly being unpatentable over Tan et al. in view of Schwartz et al. (Science 276:1872-1874, 1997). Applicants respectfully disagree.

Both references are directed towards maize proteins having neoxanthin cleavage activity. The Tan et al. reference has been discussed above. Schwartz et al. apparently is being relied upon to show that a neoxanthin cleavage activity protein (i.e., VP14) functions in the biosynthesis of ABA. But the latter reference does nothing to cure the above-noted deficiencies of Tan et al. Therefore, the combination does not produce the claimed invention.

Withdrawal of the 35 U.S.C. 103 rejections is respectfully requested.

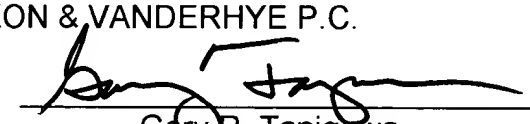
Conclusion

Having fully responded to all of the pending objections and rejections contained in the Office Action (Paper No. 17), Applicants submit that the claims are in condition for

allowance and earnestly solicit an early Notice to that effect. The Examiner is invited to contact the undersigned if any further information or claim amendments are required.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: 

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APPENDIX
MARKED-UP VERSION TO SHOW CHANGES

IN THE CLAIMS

The claims are amended as follows.

1. (Amended) ~~A~~ An isolated DNA encoding a protein having neoxanthin cleavage activity ~~for improving stress tolerance in a plant~~, wherein said protein is selected from the group consisting of:

(a) a protein comprising the amino acid sequence of SEQ ID NO:6;

(b) a protein encoded by a gene that hybridizes under stringent conditions with the nucleotide sequence of SEQ ID NO:5, wherein said stringent conditions are: (i) hybridization in a solution containing 30% formamide, 6X SSC, 5X Denhardt's solution, and 100 µg/ml denatured salmon sperm DNA at 37°C and (ii) washing in 1X SSC and 1% SDS at room temperature for 15 min;

(c) a protein comprising an amino acid sequence of SEQ ID NO:6 having up to ten conservative amino acid substitutions; and

(d) a protein comprising an amino acid sequence that is at least 80% identical to the sequence set forth in SEQ ID NO:6.

5. (Amended) A ~~transformant~~ transformed plant cell ~~carrying~~ comprising the isolated DNA of claim 1.

6. (Amended) A transgenic plant comprising the ~~transformant~~ transformed plant cell of claim 5.

7. (Amended) A transgenic plant which is an offspring or a clone of the ~~transformant~~ transgenic plant of claim 6, wherein plant cells from said offspring or clone also contain the isolated DNA which encodes a protein having neoxanthin cleavage activity and said protein is selected from the group consisting of:

(a) a protein comprising the amino acid sequence of SEQ ID NO:6;

(b) a protein encoded by a gene that hybridizes under stringent conditions with the nucleotide sequence of SEQ ID NO:5, wherein said stringent conditions are: (i) hybridization in a solution containing 30% formamide, 6X SSC, 5X Denhardt's solution, and 100 µg/ml denatured salmon sperm DNA at 37°C and (ii) washing in 1X SSC and 1% SDS at room temperature for 15 min;

(c) a protein comprising the amino acid sequence of SEQ ID NO:6 having up to ten conservative amino acid substitutions; and

(d) a protein comprising an amino acid sequence that is at least 80% identical to the sequence set forth in SEQ ID NO:6.

8. (Amended) The transgenic plant of claim 6, wherein the expression of ~~a gene~~ the isolated DNA encoding a protein having a neoxanthin cleavage activity is increased or decreased compared ~~with its~~ to the expression level in the wild type of said transgenic plant.

9. (Amended) The transgenic plant of ~~any one of~~ claim 6, wherein the amount of abscisic acid is increased or decreased compared ~~with its~~ to the wild type of said transgenic plant.

10. (Amended) The transgenic plant of claim 6, wherein stress tolerance is increased or decreased compared ~~with its~~ to the wild type of said transgenic plant.

13. (Amended) A method for producing the transgenic plant ~~carrying~~ comprising the isolated DNA of claim 1, comprising the steps of introducing said isolated DNA into a plant cell and regenerating a plant from the plant cell.

14. (Amended) A method for increasing or decreasing stress tolerance in a plant, ~~comprising expressing the DNA of claim 1 in a plant cell~~ wherein said method comprises ~~the steps of~~:

(a) introducing an isolated DNA encoding a protein having neoxanthin cleavage activity into a plant cell obtained from said plant;

(b) expressing the isolated DNA in said plant cell; and

(c) producing a plant from the plant cell that has decreased or increased stress tolerance.

Claims 2-4 have been canceled without prejudice or disclaimer.

New claims 15-22 have been added.

the manufacturer. The coding sequence for dCtBP, containing codons 8 to 383, was inserted into the pGEX-5X-3 expression plasmid (Pharmacia) and transformed into strain BL21::DE3 pLysS of *Escherichia coli*. Protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 3 hours. Cells were then sonicated in buffer A [20 mM tris-HCl (pH 7.9), 0.2 mM EDTA, 0.1 M NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.2% Nonidet P-40], and total lysates containing the GST and GST-dCtBP proteins were immobilized onto glutathione-agarose beads (Sigma) in buffer A. Binding assays were done as described (*29*). ^{35}S-labeled proteins were fractionated on a 10% SDS-polyacrylamide gel and visualized by autoradiography.

19. D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988).
20. A. C. Spradling *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10824 (1995).
21. Genomic DNA was extracted from l(3)03463 heterozygous stocks, annealed with various primers, and then subjected to polymerase chain reaction (PCR) amplification using standard methods. A 550-bp DNA fragment was obtained using a 23-nucleotide primer from the 5′ end of the dCtBP cDNA (TGAAAGCTGCGAGTGGAATTTGG) and a 28-nucleotide primer from the 3′ region of the P-element (CTTGCCGACGGGACCACCTTATGTTATT). The 5′ end of this PCR product contains 37 bp of perfect identity to the 5′ end of the dCtBP UTR. The remaining sequence does not contain any discernible homology with dCtBP, which suggests that the dCtBP UTR is interrupted by an intron located 37 bp downstream of the 5′ end of the largest dCtBP cDNA. It would appear that the P-element maps within this intron.
22. N. Perrimon, A. C. Lanjuin, C. Arnold, E. Noll, *Genetics* **144**, 1681 (1996). Additional information about l(3)03463/dCtBP can be obtained in Flybase (http://flybase.bio.indiana.edu/.bin/fbidq.html?FBal0009468).
23. G. Tearle and C. Nusslein-Volhard, *Dros. Inform. Serv.* **66**, 209 (1987).
24. P-elements were introduced into the *Drosophila* germline by injection of yw^{67} or w^{1118} embryos as described (*3*). In situ hybridizations on whole mount preparations of staged, transgenic embryos were also done as described (*3*) using a digoxigenin–uridine triphosphate–labeled lacZ antisense RNA probe. The stripe 2 reporter gene and Gal4 expression vector are identical to those used by Arnosti *et al.* (*8*).
25. W. Hanna-Rose, J. D. Licht, U. Hansen, *Mol. Cell. Biol.* **17**, 4820 (1997).
26. For a review, see M. Pazin and J. T. Kadonaga, *Cell* **89**, 325 (1997).
27. Y. Yu *et al.*, *Nature* **385**, 552 (1997).
28. D. J. Sourdive, C. Transy, S. Garbay, M. Yaniv, *Nucleic Acids Res.* **25**, 1476 (1997).
29. H. Zhang, K. M. Catron, C. Abate-Shen, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1764 (1996).
30. Y. Nibu and H. Zhang, unpublished data.
31. A GST-dCtBP fusion protein containing amino acid residues 8 to 383 was injected into a rat (Pocono Rabbit Farm, PA). The preimmune serum did not detectably cross-react with fixed embryos. The GST-dCtBP antiserum specifically stained nuclei in mixed-stage embryos. Reduced staining was detected in l(3)03463 homozygous embryos. Embryos were fixed and preincubated in bovine serum albumin as described (*15*). The rat serum was diluted 1:1000, and dCtBP was visualized using a 1:200 dilution of tetramethyl rhodamine isothiocyanate–conjugated antibodies to rat immunoglobulin (Jackson Labs).
32. G. Poortinga, M. Watanabe, S. M. Parkhurst, *EMBO J.*, in press.

33. We thank D. Rio for help with PCR amplification, L. Pick for the yeast two-hybrid DNAs and the pACT cDNA expression library, and D. Rio, L. Mirels, and R. Tjian for helpful comments. Supported by NIH grant GM46638. Y.N. is supported by a postdoctoral fellowship from the Uehara Memorial Foundation.

31 December 1997; accepted 6 March 1998

Arabidopsis CBF1 Overexpression Induces *COR* Genes and Enhances Freezing Tolerance

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Many plants, including *Arabidopsis*, show increased resistance to freezing after they have been exposed to low nonfreezing temperatures. This response, termed cold acclimation, is associated with the induction of *COR* (cold-regulated) genes mediated by the C-repeat/drought-responsive element (CRT/DRE) DNA regulatory element. Increased expression of *Arabidopsis* CBF1, a transcriptional activator that binds to the CRT/DRE sequence, induced *COR* gene expression and increased the freezing tolerance of nonacclimated *Arabidopsis* plants. We conclude that CBF1 is a likely regulator of the cold acclimation response, controlling the level of *COR* gene expression, which in turn promotes tolerance to freezing.

Studies of the molecular basis of plant tolerance to freezing have focused primarily on the cold acclimation response, the process by which plants increase their tolerance to freezing in response to low nonfreezing temperatures (*1*). Cold acclimation is associated with biochemical and physiological changes and alterations in gene expression (*1*, *2*). Studies of genes stimulated by low temperature have revealed that many, including the *Arabidopsis* COR genes, encode hydrophilic polypeptides that potentially promote tolerance to freezing (*1–3*). Indeed, constitutive expression of *COR15a* (which encodes the chloroplast-targeted polypeptide COR15am) in transgenic *Arabidopsis* plants improves the freezing tolerance of chloroplasts frozen in situ and of protoplasts frozen in vitro (*4*). Unlike cold acclimation, however, *COR15a* expression has no discernible effect on the survival of frozen plants (*2*, *5*).

Genetic analyses indicate that multiple genes are involved in cold acclimation in plants (*6*). Several COR genes are coordinately stimulated along with *COR15a* in response to low temperature (*2*, *7*), which suggests that *COR15a* might act in concert with other *COR* genes to enhance tolerance to freezing in plants. If so, expression of the entire battery of COR genes would have a greater effect on freezing tolerance than *COR15a* expression alone. To test this hypothesis, we attempted to induce expression of the COR gene "regulon" with the *Arabidopsis* transcriptional activator CBF1 (CRT/DRE binding factor 1) (*8*), a putative COR gene regulator. CBF1 binds to the cis-acting CRT (C-repeat)/DRE (drought-responsive element) sequence (*9*, *10*), a DNA regulatory element that stimulates transcription in response to both low temperature and water deficit (*9*). The element is present in the promoters of multiple COR genes including *COR15a*, *COR78* (also known as RD29A and LTI78), and *COR6.6* (*10–12*). Expression of *CBF1* in yeast (*Saccharomyces cerevisiae*) activates expression of reporter genes that have the CRT/DRE as an upstream activator sequence (*8*).

We created transgenic *Arabidopsis* plants that overexpress *CBF1* by placing a cDNA encoding CBF1 under the control of the strong cauliflower mosaic virus (CaMV) 35S RNA promoter and transforming the chimeric gene into *Arabidopsis* ecotype RLD plants (*13*). Initial screening gave rise to two transgenic lines, A6 and B16, that accumulated *CBF1* transcripts at high concentrations. Southern blot analysis indicated that the A6 plants had a single DNA insert and the B16 plants had multiple inserts. Examination of fourth generation homozygous A6 and B16 plants indicated that amounts of *CBF1* transcript were higher in nonacclimated A6 and B16 plants than they were in nonacclimated RLD plants (Fig. 1A). Quantities of *CBF1* transcript were greater in the A6 plants than in the B16 plants (Fig. 1A).

CBF1 overexpression induced COR gene expression without a low-temperature stimulus (Fig. 1A). Specifically, greater than normal amounts of *COR6.6*, *COR15a*, *COR47*, and *COR78* transcripts were detected in nonacclimated A6 and B16 plants. In nonacclimated A6 plants, COR transcript concentrations approximated those found in cold-acclimated RLD plants. In nonacclimated B16 plants, they were less than in cold-acclimated RLD plants. Immunoblot analysis indicated that the amounts of the COR15am (Fig. 1B) and COR6.6 polypeptides were also elevated in the A6 and B16 plants, with a higher level of expression in A6 plants. We were unable to identify the CBF1 protein in either RLD or transgenic plants (*5*). Overexpression of *CBF1* did not affect transcript concentrations of *eIF4A*

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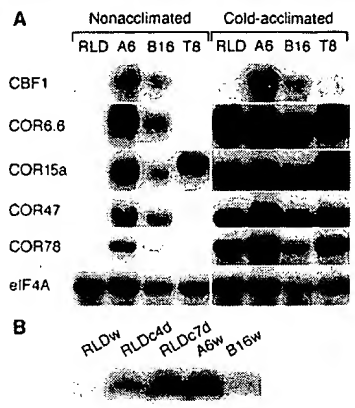

Fig. 1. Expression of *CBF1* and *COR* genes in RLD and transgenic *Arabidopsis* plants. (**A**) *CBF1* and *COR* transcripts. Leaves from nonacclimated and 3-day cold-acclimated plants (*20*) were harvested and total RNA was prepared and analyzed for *CBF1* and *COR* transcripts by RNA blot analysis with ^{32}P-radiolabeled probes (*21*). The autoradiograms for *CBF1* resulted from 3-day film exposure and those for *COR6.6* and *COR15a* were from a 3-hour exposure (the ^{32}P-radiolabeled probes were of similar specific activity). (**B**) COR15am proteins. Total soluble protein (100 μg) was prepared from leaves of the nonacclimated RLD (RLDw), 4-day cold-acclimated RLD (RLDc4d), 7-day cold-acclimated RLD (RLDc7d), and nonacclimated A6 and B16 plants; the amounts of COR15am were determined by immunoblot analysis with antiserum raised against the COR15am polypeptide (*22*). No reacting bands were observed with preimmune serum.

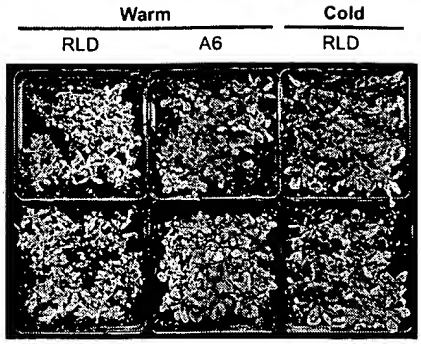

Fig. 3. Freezing survival of RLD and A6 *Arabidopsis* plants. Nonacclimated (Warm) RLD and A6 plants and 5-day cold-acclimated (Cold) RLD plants were frozen at −5°C for 2 days and then returned to a growth chamber at 22°C (*24*). A photograph of the plants after 7 days of regrowth is shown.

(eukaryotic initiation factor 4A) (*14*), a constitutively expressed gene that is not responsive to low temperature (Fig. 1A), and had no obvious effects on plant growth and development.

Two additional transgenic lines, K16 and 1-11, that overexpress *CBF1* have recently been identified. Northern blot analysis of nonacclimated T2 generation plants indicated that, in both of these lines, *COR* gene expression is also higher than that in nonacclimated RLD plants.

CBF1 overexpression increased the tolerance of plants to freezing (Fig. 2), as determined by the electrolyte leakage test (*15*).

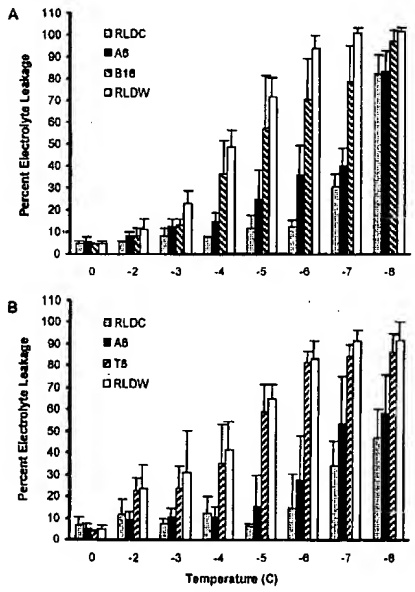

Fig. 2. Freezing tolerance of leaves from RLD and transgenic *Arabidopsis* plants. Leaves from nonacclimated RLD (RLDw) plants, 10-day cold-acclimated RLD (RLDc) plants, and nonacclimated A6, B16, and T8 plants were frozen at the indicated temperatures and the extent of cellular damage was estimated by measuring electrolyte leakage (*23*). Error bars indicate standard deviations.

Detached leaves were frozen to various subzero temperatures and, after thawing, cellular damage (due to freeze-induced membrane lesions) was estimated by measuring ion leakage from the tissues. Leaves from nonacclimated A6 and B16 plants were more tolerant to freezing than those from nonacclimated RLD plants (Fig. 2). The freezing tolerance of leaves from nonacclimated A6 plants exceeded that of leaves from nonacclimated B16 plants (Fig. 2A), which had lower levels of *CBF1* and *COR* gene expression (Fig. 1A). T8 transgenic plants (*4*), which constitutively express only *COR15a* (under control of the CaMV 35S RNA promoter) (Fig. 1A), were less freezing tolerant than A6 plants (Fig. 2B).

A comparison of EL_{50} values (the freezing temperature that results in release of 50% of tissue electrolytes) of leaves from RLD, A6, B16, and T8 plants is presented in Table 1. Data from multiple experiments indicate that the freezing tolerance of leaves from nonacclimated A6 and B16 plants was greater than that of leaves from nonacclimated RLD and T8 plants and that leaves from nonacclimated A6 plants were more freezing tolerant than leaves from nonacclimated B16 plants.

The enhancement of freezing tolerance in A6 plants was apparent in whole plant survival tests (Fig. 3). Nonacclimated A6 plants displayed variable, but greater, freezing tolerance than nonacclimated RLD plants (Fig. 3). No difference in plant survival was detected between nonacclimated B16 and RLD plants and nonacclimated T8 and RLD plants.

Our results demonstrate that constitutive overexpression of the *Arabidopsis* transcriptional activator CBF1 induces expression of *Arabidopsis COR* genes and increases the freezing tolerance of nonacclimated plants. These results are consistent with CBF1 having a role in regulating *COR* gene expression and further link the *COR* genes to plant cold acclimation. The increase in freezing tolerance brought about by expressing the battery of CRT/DRE-regulated *COR* genes was greater than that brought about by overexpressing *COR15a* alone, which implicates

Table 1. Comparison of EL_{50} values of leaves from RLD and transgenic *Arabidopsis* plants. EL_{50} values were calculated and compared by analysis of variance (*25*). EL_{50} ± SE (*n*) are presented on the diagonal line for leaves from nonacclimated RLD (RLDw), cold-acclimated (7 to 10 days) RLD (RLDc), and nonacclimated A6, B16, and T8 plants. *P* values for comparisons of EL_{50} values are indicated in the intersecting cells.

	EL_{50} values				
	RLDw	RLDc	A6	B16	T8
RLDw	−3.9 ± 0.21 (8)	$P < 0.0001$	$P < 0.0001$	$P = 0.0014$	$P = 0.7406$
RLDc		−7.6 ± 0.30 (4)	$P = 0.3261$	$P < 0.0001$	$P < 0.0001$
A6			−7.2 ± 0.25 (6)	$P < 0.0001$	$P < 0.0001$
B16				−5.2 ± 0.27 (5)	$P = 0.0044$
T8					−3.8 ± 0.35 (3)

additional *COR* genes in freezing tolerance. Whether CRT/DRE-containing *COR* genes are involved in bringing about the full array of biochemical and physiological changes that occur with cold acclimation (*1*, *2*) remains to be determined.

Freezing temperatures greatly limit the geographical distribution of native and cultivated plants and often cause severe losses in agricultural productivity (*16*). Traditional plant breeding approaches have met with limited success in improving the freezing tolerance of agronomic plants (*6*). The freezing tolerance of the best wheat varieties today is essentially the same as the most freezing-tolerant varieties developed in the early part of this century. Biotechnology, however, may offer new strategies. Here we show that the freezing tolerance of nonacclimated *Arabidopsis* plants is enhanced by increasing the expression of the *Arabidopsis* regulatory gene *CBF1*. The CRT/DRE DNA regulatory element we have targeted here is not limited to *Arabidopsis* (*17*) and thus may provide a way to improve the freezing tolerance of crop plants.

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13. Standard procedures were used for plasmid manipulations (*18*). The CBF1-containing Ase I–Bgl II fragment from pACT-Bgl+ (*8*) was gel purified, Bam HI linkers were ligated to both ends, and the fragment was inserted into the Bam HI site in pCIB710 [S. Rothstein *et al.*, *Gene* **53**, 153 (1987)], which contains the CaMV 35S RNA promoter and terminator. The chimeric plasmid was linearized at the Kpn I site and inserted into the Kpn I site of the binary vector pCIB10g (Ciba-Geigy, Research Triangle Park, NC). The plasmid was transformed into *Agrobacterium tumefaciens* strain C58C1(pMP90) by electroporation. *Arabidopsis* plants were transformed by the vacuum infiltration procedure [N. Bechtold, J. Ellis, G. Pelletier, *C. R. Acad. Sci. Ser. III Life Sci.* **316**, 1194 (1993)] as modified [A. van Hoof and P. J. Green, *Plant J.* **10**, 415 (1996)].
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20. *Arabidopsis thaliana* ecotype RLD plants were grown in pots under continuous light (~100 μE m^{-2} s^{-1}) at 22°C for 18 to 25 days as described (*19*). In some cases, plants were then cold acclimated at 2.5°C under continuous light (~50 μE m^{-2} s^{-1}) for various amounts of time.
21. Total RNA was isolated from plant leaves and subjected to RNA blot analysis by high-stringency hybridization and wash conditions as described (*8*, *19*). DNA probes were gel purified and radiolabeled with ^{32}P by random priming according to standard procedures (*18*).
22. Total soluble protein was isolated from plant leaves, fractionated by tricine SDS–polyacrylamide gel electrophoresis, and transferred to 0.2-μm nitrocellulose as described (*4*). COR15am protein was detected with antiserum raised to purified COR15am and protein A–conjugated alkaline phosphatase (Sigma) (*4*).
23. Electrolyte leakage tests were conducted as described (*15*, *19*) with the following modifications. Two to four detached leaves from nonacclimated or cold-acclimated plants were placed in a test tube and submerged for 1 hour in a −2°C bath containing water and ethylene glycol in a completely randomized design, after which ice crystals were added to nucleate freezing. After an additional hour of incubation at −2°C, the samples were cooled in decrements of 1°C each hour. Samples (five replicates for each data point) were thawed overnight on ice and incubated in 3 ml of distilled water with shaking at room temperature for 3 hours. Electrolyte leakage from leaves was measured with a conductivity meter. The solution was then removed, the leaves were frozen at −80°C (for at least 1 hour), and the solution was returned to each tube and incubated for 3 hours to obtain a value for 100% electrolyte leakage.
24. Pots (9 cm) containing about 40 nonacclimated *Arabidopsis* plants (20 days old) and 4-day cold-acclimated plants (25 days old) (*20*) were placed in a completely randomized design in a −5°C cold chamber in the dark. After 1 hour, ice chips were added to each pot to nucleate freezing. Plants were removed after 2 days and returned to a growth chamber at 22°C.
25. Model curves fitting up to third-order linear polynomial trends were determined for each electrolyte leakage experiment. To ensure unbiased predictions of electrolyte leakage, trends significantly improving the model fit at the 0.2 probability level were retained. EL_{50} values were calculated from the fitted models. An unbalanced one-way analysis of variance, adjusted for the different number of EL_{50} values for each plant type, was determined by using SAS PROC GLM [SAS Institute, *SAS/STAT User's Guide, Version 6* (SAS Institute, Cary, NC, 1989)].

26. We wish to thank J. Dodgson, B. Sears, T. Deits, and E. Stockinger for critical reading of the manuscript. This research was supported in part by grants to M.F.T. from the National Science Foundation (IBN 9307348), the U.S. Department of Agriculture/National Research Initiative Competitive Grants Program (96-35100-3231), and the Michigan Agricultural Experiment Station.

14 October 1997; accepted 10 February 1998

Structural Conservation in Prokaryotic and Eukaryotic Potassium Channels

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Toxins from scorpion venom interact with potassium channels. Resin-attached, mutant K^+ channels from *Streptomyces lividans* were used to screen venom from *Leiurus quinquestriatus hebraeus*, and the toxins that interacted with the channel were rapidly identified by mass spectrometry. One of the toxins, agitoxin2, was further studied by mutagenesis and radioligand binding. The results show that a prokaryotic K^+ channel has the same pore structure as eukaryotic K^+ channels. This structural conservation, through application of techniques presented here, offers a new approach for K^+ channel pharmacology.

Scorpion toxins inhibit ion conduction through potassium channels by occluding the pore at the extracellular opening. A single toxin protein binds very specifically to a single K^+ channel to cause inhibition. The toxins are 35 to 40 amino acids in length and have a characteristic fold that is held rigidly by three disulfide bridges (*1*). They are active site inhibitors, because when they bind to the channel they interact energetically with K^+ ions in the pore (*2*–*4*). The interaction between these inhibitors and the pore of K^+ channels has been exploited to gain insights into the structure and function of K^+ channels.

Studies employing site-directed mutagenesis of the Shaker K^+ channel have mapped the scorpion toxin binding site to regions corresponding to the extracellular entryway of the K^+ channel from *Streptomyces lividans* (the KcsA channel) (*4*–*9*). Although the amino acids of the K^+ channel selectivity filter are highly conserved, the residues lining the entryway are quite variable. As if to mirror the amino acid variation at the binding site, the toxins are also highly variable in

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P.D. 04-07-1997
p. 1-2 = 2

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RT   "VP14 catalyzes the carotenoid cleavage reaction of abscisic acid
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